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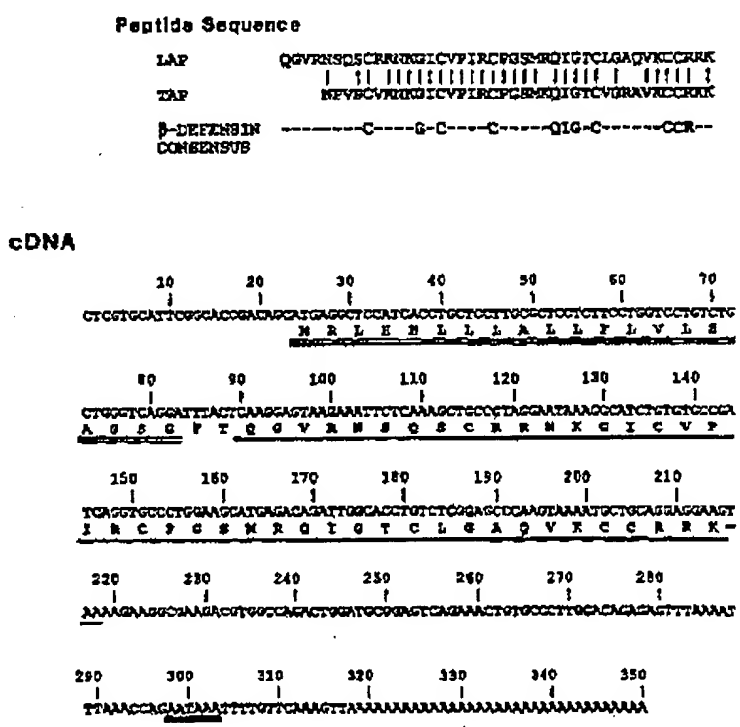
(54) Title: INDUCIBLE DEFENSIN PEPTIDE FROM MAMMALIAN EPITHELIA

(57) Abstract

The present invention relates to an inducible antimicrobial peptide designated lingual antimicrobial peptide (LAP) which has antibacterial and antifungal activity and which can be obtained from mammalian epithelium. The prepro- and the pro- precursors of LAP are also provided. The present invention also relates to cDNA encoding LAP, the prepro-precursor or the pro-lingual precursor. In addition, methods of treating microbial infection of the epithelia are provided. Such infections can be treated by contacting the epithelia with an antimicrobially effective amount of a purified mammalian epithelial LAP or by administering a component which causes endogenous production or up-regulation of LAP.



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INDUCIBLE DEFENSIN PEPTIDE FROM MAMMALIAN EPITHELIA

Description

Technical Field

The present invention relates to inducible antimicrobial and antifungal peptides of the mammalian epithelial tissue. In particular, the present invention relates to a mammalian epithelial peptide designated lingual antimicrobial peptide (LAP) and to its precursor peptides. The invention present invention also relates to cDNA segments encoding LAP and its precursor peptides, and to methods of treating microbial infection of the epithelium.

Background Art

Epithelium is a complex tissue responsible for forming an initial, physical barrier protecting the body against potentially harmful environments. Epithelial tissue covers the outer body surfaces and lines the luminal surface of the respiratory tract, the gastrointestinal tract, and the genitourinary system to protect these surfaces from exposure to the outside environment. Epithelial surfaces, therefore, serve a "defensive" function, protecting the host from the environment (Jacob and Zasloff, Ciba Foundation Symposium 186, 1994).

Antimicrobial peptides provide a second, chemical line of defense supplementing the physical barrier of the epithelial tissue surfaces. Antimicrobial peptides, produced by various tissues in the body, have antibacterial, antifungal, and antiviral activity. These peptides, which can be classified into several families, have been found in a variety of tissues from diverse species. For example, magainins have been isolated from frogs (Zasloff, M., Proc. Natl. Acad. Sci. USA 84: 5449-5453, 1987) and cecropins have been found in insects (Boman, H.G., Cell 65: 205-207, 1991). In addition, two groups of peptides within the defensin family have been identified. β-defensins have been isolated from neutrophils of cows (Selsted et al., J. of Biol. Chem. 268:

6641-6648, 1993) and from tracheal mucosa of cows (Diamond et al., Proc. Natl. Acad. Sci. USA 88: 3952-3956, 1991; and Diamond et al., Proc. Natl. Acad. Sci. USA 90: 4596-4600, 1993), while α-defensins have been isolated from neutrophils of humans (Lehrer et al., Annual Rev. Immunol. 11: 105-128, 1993) and from the epithelial-derived Paneth cells at the base of the crypts of the small intestine in murine and human GT tracts (Ouellette et al., J. Cell Biol. 108: 1687-1695, 1989; and Jones and Bevins, J. Biol. Chem. 267: 23215-23225, 1992). The antimicrobial peptides provide a second line of defense, killing bacteria and fungus pathogens which penetrate the physical barrier.

One example of epithelial tissue is the mammalian tongue which is covered by a dense stratified epithelium. The tongue is in an environment constantly exposed to various microorganisms that are part of the microbial flora of the mouth. Despite is constant exposure to microbials, invasive infections of the tongue rarely ensue even when abrasions occur on the tongue's surface. In investigating the infection resistance property of the mammalian tongue, a novel antibacterial and antifungal peptide was isolated from the extracts of bovine tongue epithelial tissue.

Disclosure of the Invention

Accordingly, it is one object of the present invention to provide an inducible antimicrobial peptide having antibacterial and antifungal activity which can be obtained from mammalian epithelium, such as bovine tongue epithelium.

It is a further object of the present invention to provide the prepro-peptide and the pro-peptide precursors of the antimicrobial peptide.

It is another object of the present invention to provide cDNA that encodes the inducible mammalian epithelium antimicrobial peptide, the prepro-peptide and the pro-peptide.

It is yet a further object of the present invention to provide a method of treating microbial infections of the epithelium and microbial infections that extend through,

beyond, or deeper in the epitheli,. such as into connective tissue or the subdermal region.

Various other objects and advantages of the present invention will be apparent from the drawings and the following description of the invention.

In one embodiment, the present invention relates to a purified inducible mammalian epithelial lingual antimicrobial peptide (LAP) having an ion mass of about 4627.5 daltons, and having antimicrobial and antifungal activity.

In another embodiment, the present invention relates to a purified prepro-lingual antimicrobial peptide (prepro-LAP) or a purified pro-lingual antimicrobial peptide (pro-LAP).

In a further embodiment, the present invention relates to a cDNA encoding a lingual antimicrobial peptide, a prepro-lingual antimicrobial peptide, or a pro-lingual antimicrobial peptide.

In yet another embodiment, the present invention relates to a method of treating microbial infection of the epithelia. The method comprises contacting the epithelia with an antimicrobially effective amount of a purified mammalian epithelial lingual antimicrobial peptide (LAP) having an ion mass of about 4627.5 daltons, and having antimicrobial and antifungal activity so that the microbial infection is inhibited.

In yet a further embodiment, the present invention relates to a method of inducing endogenous expression of lingual antimicrobial peptide (LAP) to treat microbial infections. Endogenous expression is induced by administering to a patient in need thereof, an effective amount of a component which induces the production of LAP by epithelial tissue.

In another embodiment, the present invention relates to a method of identifying endogenous up-regulators of lingual antimicrobial peptide (LAP). The method comprises contacting an epithelial cell culture with a test substance and measuring

the level of mRNA to determine whether the test substance is an up-regulator.

In a further embodiment, the present invention relates to another method of identifying endogenous up-regulators of lingual antimicrobial peptide (LAP). Up-regulators of LAP can be identified by constructing an expression vector containing a β-defensin gene promoter operably linked to a reporter gene, infecting a host cell with the expression vector, and culturing the host cell in the presence of test substances. Whether the test substance is an up-regulator is then determined by measuring the level of mRNA or reporter gene expression.

Brief Description of the Drawings

Figure 1A shows strong cation exchange chromatography of bovine tongue epithelial extracts.

Figure 1B shows a plate assay of high phase liquid column (HPLC) column fractions which was done to access antimicrobial activity on a lawn of *E. coli* D31.

Figure 1C shows a plate assay which accesses antimicrobial activity against *C. tropicalis*.

Figure 2A shows the peptide sequence of LAP, TAP, and β-defensin consensus (SEQ ID NOs:1 and 10).

Figure 2B shows the cDNA sequence of LAP.

Figure 3 shows the induction of LAP (SEQ ID NOs:11 and 12) message surrounding areas of infection. Figures A and B show normal expression of LAP mRNA in bovine tongue epithelium using in-situ hybridization while Figures C-F show representative in-situ hybridization of naturally occurring bovine tongue lesions. Figures G-H are higher powered view of Figures E-F, respectively.

Figure 4 shows the developmental expression and tissue distribution of bovine mRNA for LAP.

Best Mode for Carrying Out the Invention

The present invention relates to an inducible antimicrobial peptide designated lingual antimicrobial peptide (LAP). LAP is a mammalian antimicrobial peptide which has an ion mass of about 4627.5 daltons and possesses both

antimicrobial and antifungal activity. In one embodiment of the present invention, LAP has amino acid sequence: (SEQ ID NO:1) QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK. This peptide, obtainable from bovine epithelial tissue, is a member of the defensin family of antimicrobial peptides. LAP belongs to the β-defensin group of peptides as LAP contains the 11 conserved amino acid residues shared by all β-defensins. In addition, the signal sequence of LAP is similar to the signal sequence of the tracheal mucosa antimicrobial peptide (TAP), a β-defensin described by Diamond et al. (Diamond et al., Proc. Natl. Acad. Sci. USA 88: 3952-3956, 1991).

Antimicrobial peptides of the defensin family have been found in several species including humans, rabbits, rats, mice, and guinea pigs (Ganz et al., Med. Microbiol. Immunol. 181: 99-105, 1992; and Lehrer et al., Annual Rev. Immunol. 11: 105-128, 1993). Defensins of bovine origin have been placed in the β-defensin group while homologous defensins of human origin are designated α-defensins. As defensin peptides exist in many mammalian species, the present invention relates to all mammalian LAP including, but not limited to, LAP of bovine origin and LAP of human origin. Homologous LAPs from species other than cows can be obtained, for example, using the isolation strategy employed with the bovine tongue extracts or using cDNA probes. For example, epithelial tissue from humans could be probed using either the LAP 48-mer probe: (SEQ ID NO:2) 5'-CCT-CCT-GCA-GCA-TTT-TAC-TTG-GGC-TCC-GAG-ACA-GGT-GCC-AAT-CTG-TCT-3', or the signal sequence 51-mer probe: (SEQ ID NO:3) 5'-AGC-AGA-CAG-GAC-CAG-GAA-GAG-GAG-CGC-(AG)AG-GAG-CAG-GTG-ATG-GAG-CCT-CAT-3', or the human α-defensin signal sequence which is highly conserved (Jones and Bevins, J. Biol. Chem. 267: 23215-23225, 1992). This would identify tissue that would contain either α or β defensin. One could purify the defensin peptide from this tissue or clone the corresponding cDNA by reverse transcribing the poly-A RNA message obtained from these tissues. Alternatively, one could make a cDNA library from these tissues and then clone the corresponding cDNA from the library using the probes described

above and standard molecular biology techniques (Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (New York, 1989)).

LAP has broad spectrum antimicrobial activity against Gram-negative bacteria, Gram-positive bacterial and fungal pathogens. The peptide may also have antiviral activity. For example, LAP has a specific activity against *Escherichia coli* of 16-32 μg/ml, *Pseudomonas aeruginosa* of 63-125 μg/ml, *Staphylococcus aureus* of 63-125 μg/ml, *Candida albicans* of 32-63 μg/ml, and *Candida tropicalis* of 16-32 μg/ml.

When translated from mRNA the peptide of the present invention, LAP, begins as a prepro-precusor peptide, designated prepro-LAP. This precursor peptide contains a signal sequence consisting of about 20 amino acids followed by a short putative pro sequence consisting of about 2 amino acids. Thus, the present invention relates to the prepro-LAP and the pro-LAP precursor peptides as well as to LAP. Indeed, in one embodiment of the present invention, prepro-LAP has amino acid sequence: (SEQ ID NO:4) MRLHHLLLALLFLVLSAGSGFTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK, and in a further embodiment, pro-LAP has amino acid sequence: (SEQ ID NO:5) FTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

While the present invention is exemplified with the purification of LAP from bovine tongue epithelia, the skilled artisan will understand that the peptides of the present invention can be purified, that is isolated from proteins with which they are normally associated, from other epithelial tissues. Suitable epithelial tissues include, but are not limited to, epithelia from the respiratory tract, such as trachea, bronchi, and lung tissue, the gastrointestinal tract, such as cecum, colon, and rectum tissue, the genitourinary tract, such as bladder tissue, the reproductive tract including testes, and facial epithelia, such as conjunctiva. Further, in addition to using peptide purification methods, peptides of the present invention can be chemically synthesized or recombinantly produced using standard techniques in the art.

The present invention also relates to cDNA which encode the prepro-LAP, pro-LAP and/or LAP peptides. In particular, cDNA of the present invention include nucleotide sequences which code for an amino acid sequence selected from the group consisting of:
QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK,
MRLHHLLLALLFLVLSAGSGFTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK,
and FTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK (SEQ ID NOs:1, 4, and 5, respectively). Examples of cDNA of the present invention include, but are not limited, the nucleotide sequences of Figure 2B.

The present invention further relates to recombinant DNA molecules comprising a vector and a cDNA encoding prepro-LAP, pro-LAP or LAP. Possible vectors include, but are not limited to, Bluescript, Bluescript II, pGEM, pRIT, and PET vectors. Host cells transformed with these recombinant DNA molecules using standard techniques can be cultured to provide a source of LAP or its precursor peptides. Suitable host cells include eukaryotic and prokaryotic cells such as yeast, *E. coli*, DH5α, and HB101.

While LAP is constitutively expressed at low levels in mammalian epithelia, high levels of mRNA expression are induced in response to epithelia injury and/or infection. For example, increased concentrations of LAP mRNA are found in epithelia surrounding acute and chronic areas of infection or inflammation. This suggests that LAP plays a role in innate immunity. According to Janeway (Janeway, C.A.J. Jr., Immunology Today 13: 11-16, 1992), innate immunity is characterized by three properties: polyspecificity, ability to discern self from nonself, and rapid response kinetics. LAP of the present invention is a broad spectrum antibiotic which is polyspecific and inducible upon infection, with induction occurring rapidly enough to be present in areas of acute inflammation. These findings are consistent with each of Janeway's hypotheses and suggests that LAP plays a role in innate immunity protecting epithelia from injury and infection.

Accordingly, peptides of the present invention can be used to treat epithelial diseases and microbial infections. LAPs can be used to treat epithelial diseases such as, diseases occurring in any immunodeficiency state, cystic fibrosis, and gum diseases and wounds, as well as microbial infections of the epithelia such as, bacterial and viral infection, and infections that extend through, beyond, or deeper in the epithelial such as into the connective tissue or subdermal regions. To treat such conditions, the diseased or infected epithelial tissue is contacted an antimicrobially effective amount of LAP or a precursor of LAP, either alone or in a pharmaceutically acceptable carrier. Suitable carriers include cremes, gels, saline, water, paste, and liposomes made of phospholipids. The LAP administered in this manner can be purified from epithelial tissue, recombinantly produced using the recombinant vector of the present invention or chemically synthesized.

The effective amount of LAP will vary depending on several factors such as, for example, the severity of the disease or infection, the causative organism and the type of epithelial tissue being treated, but the amount required for a particular patient given the patient's history and symptoms is easily determinable by one skilled in the art. For example, LAP could be applied to gums with gingivitis in micromolar amounts greater than the minimum inhibitory concentration (MIC) of LAP for *Staphylococcus*. LAP could be used as an antifungal in the mouth or GI tract since it has activity against *Candida albicans* and *Candida tropicalis*, *in vitro* and can be administered in a dose that provides a local tissue level greater than the MIC for that organism or in several smaller doses that can be repeated.

In addition, in the respiratory tract one could use LAP to treat pneumonia, bronchitis, or cystic fibrosis. For example, LAP could be inhaled, aerosolized, placed in a liposome and inhaled, or lavaged into the respiratory tract. These formulations could also be used to place the LAP in contact with the genitourinary or reproductive tracts. LAP

could also be applied directly to a skin wound, burn or infection.

Lingual antimicrobial peptides or other α and β defensins can also play a role in preventing or treating diseases by inducing endogenous defenses. Components of infection, such as bacterial cell wall lipopolysaccharides, inactivated microbes, glycolipids, glycoproteins, sugars, or viral components, can be identified which induce the expression of LAP mRNA in epithelial tissues. Such components can be identified using standard techniques such as those employed by Brey et al. (Proc. Natl. Acad. Sci. USA 90: 6275-6279, 1993), and Diamond and Bevins (Chest. 1994 March 105(3 Suppl) 51s-52s, 1994). Accordingly, the present invention also provides methods of screening test substances to determine whether they are up-regulators of LAP. For example, cultures of epithelial cells capable of expressing LAP can be exposed to various components and the amount of mRNA produced by the cells measured to determine whether exposure to a given component increased the mRNA expression. Alternatively, an expression vector system could be designed with a β-defensin promoter operably linked to a reporter gene. Suitable reporter genes included chloramphenical acetyl transferase or β-galactosidase. Host cells infected with such an expression vector could be cultured in the presence of test substances and the ability of these substances to up-regulate LAP or other α and β defensins determined by measuring the level of mRNA produced by the host cell or by measuring the increase in message as a function of reporter gene expression.

Components which are shown to induce the expression of LAP mRNA can then be administered to a patient to induce therapeutic endogenous expression of LAP. The induction of endogenous LAP production can be used to treat, for example, patients with AIDS, severe microbial infections, inflammatory skin or gum lesions, or infections of any epithelial surface or infections that extend through, beyond, or deeper in the epithelial, such as into the connective tissue or subdermal regions.

For the purposes of illustrating a preferred embodiment of the present invention, in the following non-limiting examples, the lingual antimicrobial peptide (LAP) was isolated from bovine tongue epithelial tissue, the cDNA encoding LAP was isolated and sequence, and mRNA expression and tissue distribution analyzed. It is, however, to be understood that the discussion generally applies to the isolation of LAP or other defensins from any mammalian epithelium.

EXAMPLES

Purification of LAP Peptide

Using a purification scheme that involved organic extraction, gel filtration, reverse phase HPLC, and strong cation exchange HPLC, the lingual antimicrobial peptide (LAP) was purified from bovine tongue epithelial tissue.

Approximately 500 g of anterior tongue epithelial tissue was dissected from 5 freshly killed cows and frozen in liquid nitrogen. The tissue was pulverized in a blender using liquid nitrogen and extracted for 3 days at 4° C with 5 volumes of 60% acetonitrile, 1% Trifluoroacetic Acid (TFA). The sample was then centrifuged at 4° C for 15 minutes and the supernatant was extracted using 15 volumes chloroform:methanol (2:1). The upper aqueous phase was pooled, lyophilized, and resuspended in 15 ml of 25% acetonitrile, 1% TFA. The sample was then centrifuged at 4000 RPM for 15 minutes and the remaining supernatant was loaded on a 120 ml P-30 gel filtration column (Biorad, Richmond, California).

The active antimicrobial fractions were pooled and loaded onto a reverse phase HPLC C-18 column (Poly LC, Columbia, Maryland). The active fractions were the loaded onto a strong cation exchange HPLC column-PSEA (Poly LC, Columbia, Maryland) (Figure 1A) and each fraction was desalted using a C-18 Sep-pak cartridge (Waters, Milford, Massachusetts), dried overnight and assayed for activity against *E. coli* D31 and *C. tropicalis* as described below.

This peptide was the most abundant of several antimicrobial activities isolated from the bovine tongue

epithelium. The minimal inhibitory concentrations (MIC's) demonstrated broad spectrum antimicrobial activity against gram negative and gram positive bacteria, and fungal pathogens with a potency similar to magainin-II amide (Figure 1C) and comparable to other defensins previously isolated. (Diamond et al., Proc. Natl. Acad. Sci. USA 88: 3952-3956, 1991.)

Antimicrobial Assaying of LAP Peptide

Antimicrobial activity was determined during the purification process. Approximately 2.5 ml fractions from the P-30 gel filtration column were assessed after drawing fractions and taking an aliquot of the fraction and spotting that fraction on a radial diffusion plate as described by Zasloff, M. (Proc. Natl. Acad. Sci USA 84: 5449-5453, 1987) against *E. coli* D31 or fungal pathogens such as *Candida albicans*, *Candida tropicalis*, or *Staphylococcus aureus*. (See also, Zasloff et al., Proc. Natl. Acad. Sci. USA 85: 910-913, 1988.) (Figure 1B and 1C.) Briefly, the minimal inhibitory concentrations (MIC's) were assessed using a 96 well microtitre plate (Corning Glass Works, Corning, New York). Microorganisms were grown in log phase at 1/4 strength tryptics soy broth (TSB) at a density of 1×10^5/ml. The assays used 1/4 strength TSB. For each organism, dilutions of peptide were made ranging from > 500 μg/ml to 1 μg/ml using 1/4 strength TSB as a dilution buffer. Zones of bacterial growth or lack of growth were assessed under the microscope. MIC's were calculated based the lowest concentration of peptide that inhibited growth.

The results set forth below in Table 1 demonstrate that LAP has broad spectrum antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, and fungal pathogens. Indeed, the MIC's demonstrated broad spectrum antimicrobial activity against gram negative and gram positive bacteria, and fungal pathogens with a potency similar to magainin-II amide and comparable to other defensins previously isolated (Diamond et al., Proc. Natl. Acad. Sci. USA 88: 3952-3956, 1991).

Table 1

Antimicrobial Activity of LAP and Magainin II-amide

Microorganism (ATCC)	Mininum Inhibitory Concentration (μg/ml) LAP	Magainin II
Escherichia coli (D31)	16-32	13-25
Pseudomonas aeruginosa (27853)	63-125	13-25
Staphylococcus aureus (29213)	63-125	50-100
Candida albicans (14053)	32-63	50-100
Candida tropicalis (13803)	16-32	13-25

Sequencing LAP Peptide

The mass ion of LAP is 4627.5, consistent with the size and amino acid composition of a β-defensin (Figure 2A) (Selsted et al., J. of Biol. Chem. 268: 6641-6648, 1993.) The carboxyl (C) terminal sequence of approximately 20 amino acids of LAP were determined using microsequencing after digestion of the purified peptide with trypsin, followed by reduction and alkylation of cysteine residues (Figure 2). Briefly, the peptide fragments were sequenced using Edman degradation, a standard sequencing technique. The order of the sequenced fragments was determined with overlapping fragments or identifying homologous regions to TAP.

A polymerase chain reaction (PCR) based strategy was designed to complete the N-terminal sequence. After microsequencing, degenerate PCR primers were designed from the carboxyterminal region of the LAP amino acid sequence where there was no sequence homology to TAP and codon assignment of TAP was used for homologous amino acids. A non-degenerate primer was designed from the first six amino acids of the signal sequence derived from the cloning of the cDNA of TAP.

The primers were sense strand (SEQ ID NO:6) 5'-ATGAGGCTCCATCACCTG (non-degenerate) and (SEQ ID NO:7) 5'-(AG)CA(AG)CA(TC)TT(ACGT)AC(TC)TG(ACGT)GC-antisense strand (1:256 degeneracy). PCR conditions were 95° C for 1 minute, 58° C for 2 minutes, and 72° C for 3 minutes. This was followed by 72° C for 15 minutes. PCR products were run on an 1.2% agarose gel, purified with Geneclean II, and subcloned

into Bluescript vector modified to accept PCR products after linearization with EcoRV. The cDNA product was sequenced using dideoxy chain termination and was identical with the amino acid sequence of LAP derived from microsequencing.

Cloning and Sequencing of LAP Peptide cDNA

A cDNA library was generated from bovine tongue epithelial poly A(+) RNA (Stratagene Kit for λZAP library, La Jolla, California) and the cDNA for LAP was cloned and sequenced (Figure 2B). Briefly, a cDNA lambda Zap-cDNA library of tongue epithelial tissue was constructed from (2 μg) poly A(+) RNA and inserts were size selected from 0.1 kb to 3 kb. Approximately 0.5 x 10^6 phage were spread over 10 plates and there were approximately 100 positive pfu's per plate. The phage were isolated using a LAP cDNA probe derived from PCR containing the signal sequence and the peptide coding region (183 bp). Duplicate lifts to detect positives were used with Genescreen II nylon membranes (Dupont NEN, Boston, Massachusetts). The phage were plaque purified and approximately 6 positive phage were isolated, subcloned into Bluescript and sequenced using T3 and T7 primers and dideoxy chain termination. The sequence was confirmed in triplicate using sequences derived from multiple clones.

The cloned message encodes a 64 amino acid precursor, structurally similar to the prepro β-defensin, TAP (Diamond et al., Proc. Natl. Acad. Sci. USA 88: 3952-3956, 1991). The signal sequence consists of 20 amino acids followed by a short putative pro sequence consisting of 2 amino acids which could be cleaved by a dipeptidase as described for the antimicrobial mellitin (Boman et al., J. Biol. Chem. 264: 5852-5860, 1989; and Kreil, G., TIBS 15: 23-26, 1990). The mature peptide is at the C terminus of the precursor and consists of 42 amino acids followed by an in frame stop codon. The polyadenylation signal is 14 nucleotides from the poly A tail.

Expression and Distribution of LAP mRNA in Epithelia Tongue Tissue

The bovine tongue is covered by a dense parakeratinized stratified epithelium (Figure 3A). The upper

surface of the epithelium is comprised of senescent cells while the middle and basal layers represent transcriptionally active cells (Fuchs, E., J. Cell Biol. 111: 2807-2814, 1990). The basal layer of the epithelium is comprised of germinal cells. The epithelium is nourished by a connective tissue layer which forms papillae within the epithelium, and contains blood vessels and nerves. There is a striated muscular layer inferior to the connective tissue. To determine the expression and distribution pattern of LAP mRNA, bovine tongue tissue was hybridized with the LAP antisense probe.

Bovine tongue was obtained from Moyer Packing Company (MOPAC) (Souderton, Pennsylvania) using freshly slaughtered cows (Jersey Holstein and black angus species). The anterior epithelium was dissected from the underlying connective and muscle tissue and the epithelial tissue was fixed immediately using 4% paraformaldehyde, 1 x PBS. The tissue was embedded in a paraffin block and 6-8 µicron thick sections were cut and mounted on sialanated slides. The slides were maintained at -70° C.

Riboprobes were made with a full length cDNA of LAP subcloned into Bluescript, and linearlized with *Sma* and *Kpn* I enzymes for sense and antisense transcripts, respectively.

The slides were dried and fixed using standard in-situ conditions (Young et al., Neurosci. Lett. 70: 198-203, 1986) and hybridizations were carried out at 37° C with overnight incubations using 2 X 10^6 cpm/slide. The slides were washed at high stringency of 65° C, with β-mercapto-ethanol and the slides were exposed to autoradiographic film. The slides were dipped in photographic emulsion, Kodak NTB-2 prior to exposure of the emulsion to the slides for 4 1/2 weeks at 4° C. The slides were developed under standard conditions, then stained with hematoxalin and eosin, and photographed at 20-40x magnification.

Intense hybridization to the middle layers of the epithelium was seen in Figure 3B. (Figure 3A represents the sense-negative control.) The sense probe yielded no hybridization signal. In contrast, the mRNA for β tubulin

appeared to be distributed uniformly throughout the entire tissue section.

Although tongue epithelium has been identified as the major site of LAP tissue expression, the cellular pathways of processing or secretion have not yet been determined. Since the LAP precursor contains a signal sequence, it should be secreted from individual epithelium cells or into intracellular granules. LAP could be secreted in the pro form and processed post translationally as suggested for human defensins (Ganz et al., Blood 82: 641-650, 1993).

To discern the role of LAP in innate immunity, three cows with naturally occurring tongue lesions were selected. In all three cases, the lesions represented areas consisting of both acute and chronic infection and inflammation (Figure 3C-3F). In each case, destruction of the normal epithelium was noted. There were areas of acute inflammation characterized by hemorrhage and erythrocyte accumulation, infiltration of polymorphonuclear leukocytes, along with areas of more chronic inflammation characterized by infiltration of mononuclear cells. The area surrounding and including the tongue lesions were excised from the three cows and fixed in 4% paraformaldehyde/PBS prior to in-situ hybridization. In-situ hybridization was performed as described above. The lesions were hybridized with either full length riboprobes for LAP (sense and antisense) or β-tubulin (sense and antisense). All slides were exposed to emulsion for 4 1/2 weeks prior to developing.

An increase in the concentration of LAP mRNA was found in the remaining epithelia surrounding both acute and chronic areas of infection. The pattern of expression is consistent with induction of LAP mRNA in the existing cells of the epithelium surrounding the infection.

These observations parallel the experimental data of Brey et al. who showed induction of cecropin mRNA in the epithelial cell layer of silkworm larvae after epicuticular and cuticular wounding (Brey et al., Proc. Natl. Acad. Sci. USA 90: 6275-6279, 1993). Induction only occurs when the

abraded larvae are challenged with live bacteria or bacterial cell wall components. Diamond et al. showed in an *in vitro* system that TAP mRNA from primary cultured bovine tracheal epithelial cells was induced 5-fold by adding LPS to the culture medium (Diamond and Bevins, Chest 105(3 Suppl) 51s-52s, 1994). The sequences of the gene from the bovine defensin TAP, and both the cecropin and a diptherícin loci from drosophila, contain an nFκB site in the 5' region implicated in the LPS responsiveness of these genes (Diamond et al., Proc. Natl. Acad. Sci. USA 90: 4596-4600, 1993; Kapper et al., EMBO J. 12: 1561-1569, 1993; and Sun and Faye, Europ. J. Biochem. 204: 885-892, 1992).

For tissue distribution studies, epithelia from the gastrointestinal, respiratory, genitourinary, male and female reproductive tracts of cows and facial cow epithelia was employed. Northern blot were performed on bovine tissues. RNA was prepared from bovine epithelial tissue specimens taken from freshly killed cows. Tongue RNA was also obtained from mixed gestation aged fetal tongue (Moyer Packing Company, Souderton, Pennsylvania) and from 4 month old milk fed veal calves (March Farms, Souderton, Pennsylvania). The tissue was immediately frozen in liquid nitrogen. RNA was prepared after quanidinium isothiocyanate extraction followed by centrifugation of the RNA on a cesium chloride cushion. For the poly A(+) blot, RNA was isolated from 200 μg of total RNA, followed by isolation of poly (A)+ RNA using oligo dT push columns. 4 μg of poly (A)+ RNA from several tissues were electrophoresed on a 1.2% formaldehyde gel using 1x MOPS as a running buffer. Approximately 15 μg of total RNA was used from each specimen. The tissues were also run on a 1.2% formaldehyde gel. The gels were blotted using Zetabind positively charged nylon membranes, transferring the RNA using 10x SSC at pH 7.4. Hybridizations were carried out at 42° C, using standard hybridization conditions of 6x SSC, 5x Denhardt's, 20% formamide, 200 μg/ml of yeast RNA, 0.5% SDS. Probes were designed as follows:

LAP (48 mer): (SEQ ID NO:2) 5'-CCT-CCT-GCA-GCA-TTT-TAC-TTG-GGC-TCC-GAG-ACA-GGT-GCC-AAT-CTG-TCT-3'.

Signal sequence (51 mer): (SEQ ID NO:3) 5'-AGC-AGA-CAG-GAC-CAG-GAA-GAG-GAG-CGC-(AG)AG-GAG-CAG-GTG-AT' -GAG-CCT-CAT-3'.

The probes were each end labelled using Υ^{32}p-ATP to a specific activity of 1x 10^{8} CPM/μg DNA. The β-tubulin probe was the full length cDNA bovine clone and was labelled with α^{32}P dCTP using random priming to a specific activity of 1x 10^{9} CPM/μg DNA. The blots were hybridized overnight and washed at the following conditions:

LAP - 65° C, 1x SSC, 0.1% SDS; and

β tubulin - 65° C, 0.1x SSC, 0.1% SDS.

LAP mRNA (or closely homologous messages) were widely expressed in the numerous epithelial tissues of the bovine respiratory tract including trachea, bronchi, and bronchi/lung; lower gastrointestinal tract including cecum, colon, and rectum; reproductive system including testes; and facial epithelium including conjunctiva (Figure 4). The finding that LAP or a closely related message is expressed in so many epithelial tissues suggests that LAP plays a role in epithelial defense in sites in addition to the tongue.

LAP message was not expressed in the fetal tongue but was expressed after birth (Figure 4). This pattern of expression supports induction or developmental regulation. Thus, LAP mRNA appears to be expressed at a low constitutive level in normal bovine tongue after birth (Figure 3A), and is induced to higher levels of expression in response to injury and infection.

It is possible that LAP contributes to wound healing and/or playing a role in limiting the physical area of the infection and sterilize the tissue. Both mechanisms have been suggested previously for other defensins (Lehrer et al., Annual Rev. Immunol. 11: 105-128, 1993).

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Magainin Pharmaceuticals Inc.
5110 Campus Drive
Plymouth Meeting, PA 19462

(ii) TITLE OF INVENTION: Inducible Defensin Peptide From Mammalian Epithelia

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Finnegan, Henderson, Farabow, Garrett & Dunner
(B) STREET: 1300 I Street, N.W.
(C) CITY: Washgington
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20005-3315

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/248,016
(B) FILING DATE: 24-MAY-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ogden, Stasia L.
(B) REGISTRATION NUMBER: 36,228
(C) REFERENCE/DOCKET NUMBER: 05387.0017-00000

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-408-4000
(B) TELEFAX: 202-408-4400

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Gln Gly Val Arg Asn Ser Gln Ser Cys Arg Arg Asn Lys Gly Ile Cys
1               5                   10                  15

Val Pro Ile Arg Cys Pro Gly Ser Met Arg Gln Ile Gly Thr Cys Leu
            20                  25                  30

Gly Ala Gln Val Lys Cys Cys Arg Arg Lys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CCTCCTGCAG CATTTTACTT GGGCTCCGAG ACAGGTGCCA ATCTGTCT                48
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 28
(D) OTHER INFORMATION: /note= "N is either A or G."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AGCAGACAGG ACCAGGAAGA GGAGCGCNAG GAGCAGGTGA TGGAGCCTCA T            51
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Arg Leu His His Leu Leu Leu Ala Leu Leu Phe Leu Val Leu Ser
1               5                   10                  15

Ala Gly Ser Gly Phe Thr Gln Gly Val Arg Asn Ser Gln Ser Cys Arg
            20                  25                  30

Arg Asn Lys Gly Ile Cys Val Pro Ile Arg Cys Pro Gly Ser Met Arg
        35                  40                  45

Gln Ile Gly Thr Cys Leu Gly Ala Gln Val Lys Cys Cys Arg Arg Lys
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Phe Thr Gln Gly Val Arg Asn Ser Gln Ser Cys Arg Arg Asn Lys Gly
1               5                   10                  15

Ile Cys Val Pro Ile Arg Cys Pro Gly Ser Met Arg Gln Ile Gly Thr
            20                  25                  30

Cys Leu Gly Ala Gln Val Lys Cys Cys Arg Arg Lys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGAGGCTCC ATCACCTG 18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(1, 4)
(D) OTHER INFORMATION: /note= "N is A or G."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(7, 13)
(D) OTHER INFORMATION: /note= "N is T or C."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(10, 16)
(D) OTHER INFORMATION: /note= "N is A,C,G, or T."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

NCANCANTTN ACNTGNGC 18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAAGGAGTAA GAAATTCTCA AAGCTGCCGT AGGAATAAAG GCATCTGTGT GCCGATCAGG 60

TGCCCTGGAA GCATGAGACA GATTGGCACC TGTCTCGGAG CCCAAGTAAA ATGCTGCAGG 120

AGGAAGT 127

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTACTCAAG GAGTAAGAAA TTCTCAAAGC TGCCGTAGGA ATAAAGGCAT CTGTGTGCCG 60

ATCAGGTGCC CTGGAAGCAT GAGACAGATT GGCACCTGTC TCGGAGCCCA AGTAAAATGC

TGCAGGAGGA AGT [illegible]

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asn Pro Val Ser Cys Val Arg Asn Lys Gly Ile Cys Val Pro Ile Arg
1               5                   10                  15

Cys Pro Gly Ser Met Lys Gln Ile Gly Thr Cys Val Gly Arg Ala Val
            20                  25                  30

Lys Cys Cys Arg Lys Lys
        35
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 330 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
CTCGTGCATT CGGCACCGAC AGCATGAGGC TCCATCACCT GCTCCTTGCG CTCCTCTTCC      60
TGGTCCTGTC TGCTGGGTCA GGATTTACTC AAGGAGTAAG AAATTCTCAA AGCTGCCGTA     120
GGAATAAAGG CATCTGTGTG CCGATCAGGT GCCCTGGAAG CATGAGACAG ATTGGCACCT     180
GTCTCGGAGC CCAAGTAAAA TGCTGCAGGA GGAAGTAAAA GAAGGCGAAG ACGTGGCCAG     240
ACTGGATGCG GAGTCAGAAA CTGTGCCCTT GGACAGAGAG TTTAAAATTT AAACCAGAAT     300
AAATTTTGTT CAAAGTTAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA                350
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Arg Leu His His Leu Leu Leu Ala Leu Leu Phe Leu Val Leu Ser
1               5                   10                  15

Ala Gly Ser Gly Phe Thr Gln Gly Val Arg Asn Ser Gln Ser Cys Arg
            20                  25                  30

Arg Asn Lys Gly Ile Cys Val Pro Ile Arg Cys Pro Gly Ser Met Arg
        35                  40                  45

Gln Ile Gly Thr Cys Leu Gly Ala Gln Val Lys Cys Cys Cys Arg Arg
    50                  55                  60

Lys
65
```

CLAIMS:

1. A purified mammalian epithelial lingual antimicrobial peptide (LAP) having an ion mass of about 4627.5 daltons, and having antimicrobial and antifungal activity.

2. The purified lingual antimicrobial peptide of claim 1 having specific activity of about 16-125 μg/ml against Gram-negative bacteria, Gram-positive bacteria, and fungal pathogens.

3. The purified lingual antimicrobial peptide of claim 2 having specific activity against *Escherichia coli* of 16-32 μg/ml, *Pseudomonas aeruginosa* of 63-125 μg/ml, *Staphylococcus aureus* of 63-125 μg/ml, *Candida albicans* of 32-63 μg/ml, and *Candida tropicalis* of 16-32 μg/ml.

4. The purified lingual antimicrobial peptide of claim 3 having amino acid sequence: (SEQ ID NO:1) QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

5. The purified lingual antimicrobial peptide of claim 1 which is of bovine origin.

6. The purified lingual antimicrobial peptide of claim 1 which is of human origin.

7. A purified prepro-lingual antimicrobial peptide (prepro-LAP) having amino acid sequence: (SEQ ID NO:4) MRLH-LLLALLLVLSAGSGFTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

8. A purified pro-lingual antimicrobial peptide (pro-LAP) having amino acid sequence: (SEQ ID NO:5) FTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

9. A cDNA encoding a lingual antimicrobial peptide (LAP) which is present in mammalian epithelium having an ion mass of about 4627.5 daltons, and antimicrobial and antifungal activity.

10. The cDNA of claim 9 encoding amino acid sequence: (SEQ ID NO:1) QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

11. The cDNA of claim 10 having nucleotide sequence: (SEQ ID NO:8) CAAGGAGTAAGAAATTCTCAAAGCTGCCGTAGGAATAAA-GGCATCTGTGTGCCGATCAGGTGCCCTGGAAGCATGAGACAGATTGGCACCTGTCTCGGAG-CCCAAGTAAAATGCTGCAGGAGGAAGT.

12. A cDNA encoding a prepro-lingual antimicrobial peptide having amino acid sequence: (SEQ ID NO:4) MRLHHLLL-LLFLVLSAGSGFTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

13. The cDNA of claim 12 having nucleotide sequence set forth in Figure 2B.

14. A cDNA encoding a pro-lingual antimicrobial peptide having amino acid sequence: (SEQ ID NO:5) FTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

15. The cDNA of claim 14 having nucleotide sequence of: (SEQ ID NO:9) TTTACTCAAGGAGTAAGAAATTCTCAAAGCTGCCGTAGGAATAAA-GGCATCTGTGTGCCGATCAGGTGCCCTGGAAGCATGAGACAGATTGGCACCTGTC-TCGGAGCCCAAGTAAAATGCTGCAGGAGGAAGT.

16. A method of treating microbial infection of the epithelia comprising contacting said epithelia with an antimicrobially effective amount of a purified mammalian epithelial lingual antimicrobial peptide (LAP) having an ion mass of about 4627.5 daltons, and having antimicrobial and antifungal activity so that the microbial infection is inhibited.

17. The method of claim 16 wherein said LAP has the amino acid sequence: (SEQ ID NO:1) QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK.

18. A method of identifying endogenous up-regulators of lingual antimicrobial peptide (LAP) comprising contacting an epithelial cell culture with a test substance and measuring the level of mRNA to determine whether the test substance is an up-regulator.

19. A method of identifying endogenous up-regulators of lingual antimicrobial peptide (LAP) comprising the steps of:

i. constructing an expression vector containing a α- or β-defensin gene promoter operably linked to a reporter gene;

ii. infecting a host cell with the expression vector;

iii. culturing the host cell in the presence of test substances; and

iv. measuring the level of mRNA or reporter gene expression to determine whether the test substance is an up-regulator.

20. The method of claim 19 wherein said reporter gene is chloramphenical acetyl transferase or β-galactosidase.

21. A method of inducing endogenous expression of lingual antimicrobial peptide (LAP) to treat microbial infections, which method comprises administering to a patient in need thereof an effective amount of a component which induces the production of LAP by epithelial tissue.

22. The method of claim 21 wherein said component is LPS, an inducer of LPS, a bacterial component, or a viral component.

23. The method of claim 22 wherein said inducer of LPS is a phorbolester, a sugar, a phospholipid, or trama.

24. The method of claim 23 wherein said sugar is a glycolipid or a glycoprotein.

1/6

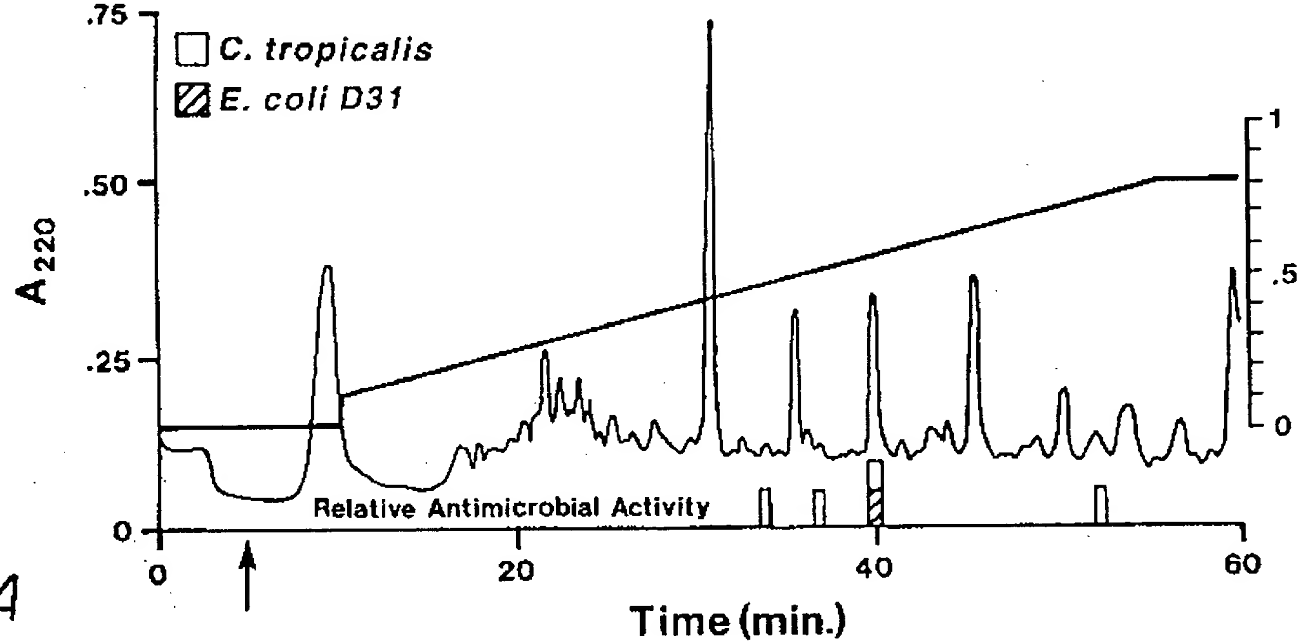


FIG. 1A

Peptide Sequence

```
LAP          QGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK
                 |  || ||||||||||||||| ||||| |  ||||| |
TAP              NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK

β-DEFENSIN   --------C----G-C----C-----QIG-C------CCR--
CONSENSUS
```

FIG. 2A

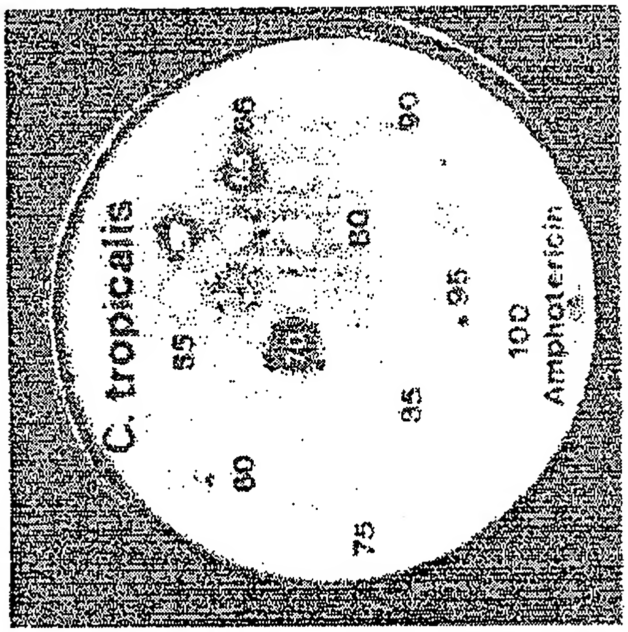

C. tropicalis
55
60
75
80
85
90
95
100
Amphotericin

FIG. 1C

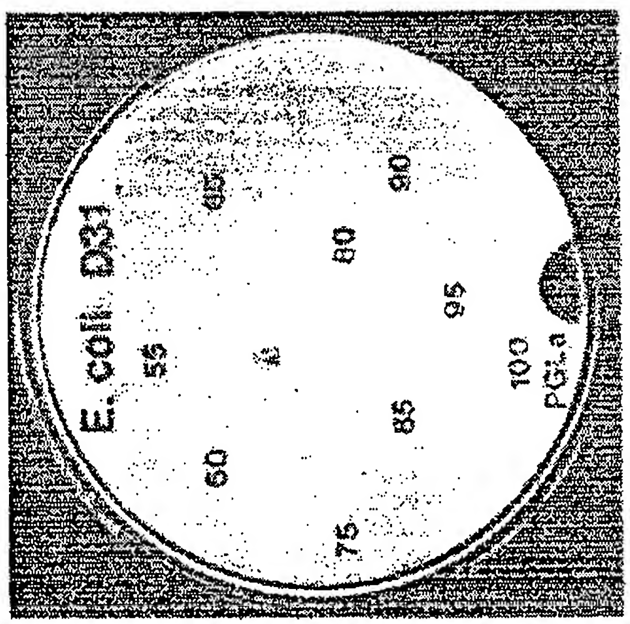

E. coli D31
55
60
65
75
80
85
90
95
100
PGLa

FIG. 1B

cDNA

```
        10        20        30        40        50        60        70
         |         |         |         |         |         |         |
CTCGTGCATTCGGCACCGACAGCATGAGGCTCCATCACCTGCTCCTTGCGCTCCTCTTCCTGGTCCTGTCTG
                         M  R  L  H  H  L  L  L  A  L  L  F  L  V  L  S

       80        90       100       110       120       130       140
        |         |         |         |         |         |         |
CTGGGTCAGGATTTACTCAAGGAGTAAGAAATTCTCAAAGCTGCCGTAGGAATAAAGGCATCTGTGTGCCGA
A  G  S  G  F  T  Q  G  V  R  N  S  Q  S  C  R  R  N  K  G  I  C  V  P

      150       160       170       180       190       200       210
        |         |         |         |         |         |         |
TCAGGTGCCCTGGAAGCATGAGACAGATTGGCACCTGTCTCGGAGCCCAAGTAAAATGCTGCAGGAGGAAGT
I  R  C  P  G  S  M  R  Q  I  G  T  C  L  G  A  Q  V  K  C  C  R  R  K  -

     220       230       240       250       260       270       280
       |         |         |         |         |         |         |
AAAAGAAGGCGAAGACGTGGCCAGACTGGATGCGGAGTCAGAAACTGTGCCCTTGGACAGAGAGTTTAAAAT

     290       300       310       320       330       340       350
       |         |         |         |         |         |         |
TTAAACCAGAATAAATTTTGTTCAAAGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

FIG. 2B

FIG. 3A

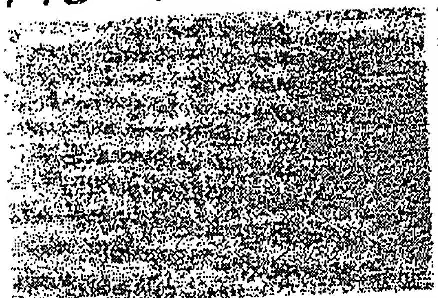


FIG. 3B

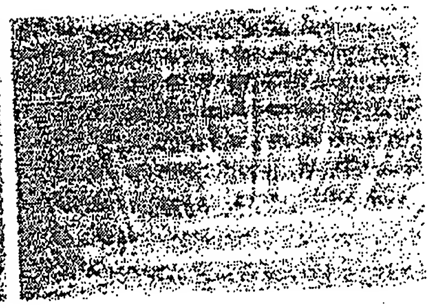


FIG. 3C

FIG. 3D

FIG. 3E

FIG. 3F

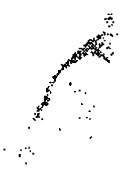
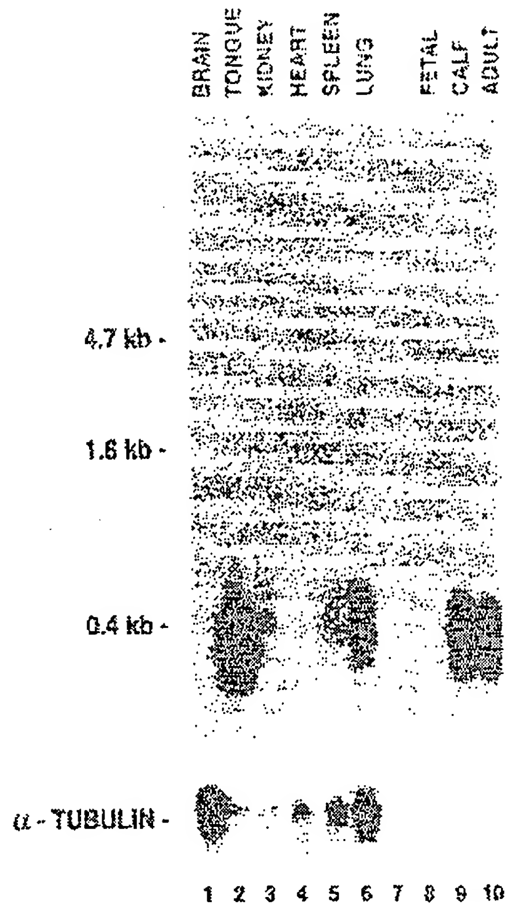


FIG. 3G

FIG. 3H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TONGUE
KIDNEY
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SPLEEN
LUNG
FETAL
CALF
ADULT
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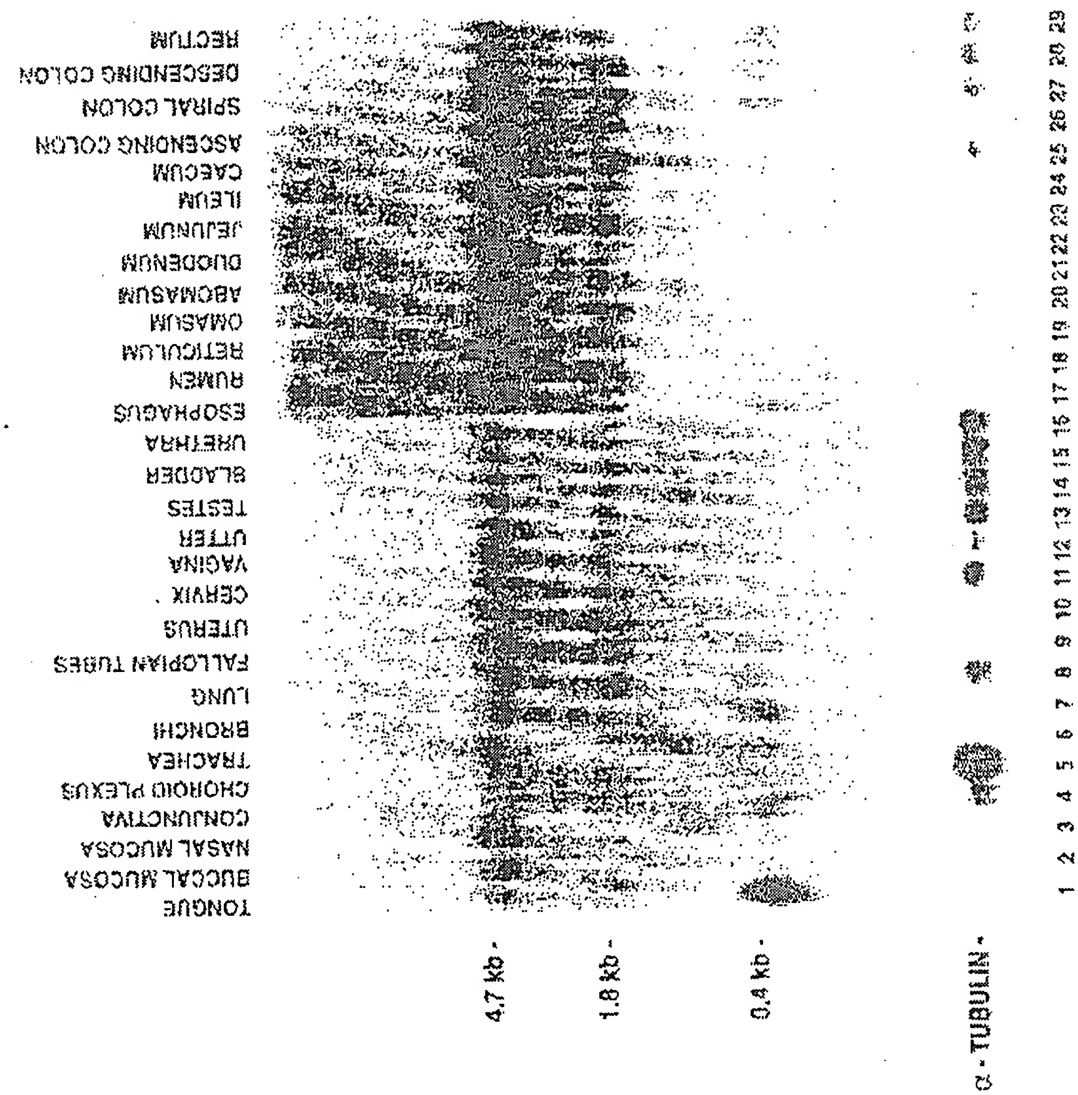
1.6 kb -
0.4 kb -
α - TUBULIN -
1 2 3 4 5 6 7 8 9 10

FIG. 4A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ASCENDING COLON
CAECUM
ILEUM
JEJUNUM
DUODENUM
ABOMASUM
OMASUM
RETICULUM
RUMEN
ESOPHAGUS
URETHRA
BLADDER
TESTES
UTTER
VAGINA
CERVIX
UTERUS
FALLOPIAN TUBES
LUNG
BRONCHI
TRACHEA
CHOROID PLEXUS
CONJUNCTIVA
NASAL MUCOSA
BUCCAL MUCOSA
TONGUE
4.7 kb -
1.8 kb -
0.4 kb -
α - TUBULIN -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

FIG. 4B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/06761

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A61K38/17 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 07873 (THE CHILDREN'S HOSPITAL OF PHILADELPHIA) 14 May 1992 see page 10, line 13 - line 24 see page 8, line 36 - page 10, line 6 see page 7, line 31 - page 8, line 24 --- -/--	1-5,7-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 1995	28.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, no. 9, 1 May 1991 WASHINGTON US, pages 3952-3956, GILL DIAMOND ET AL. 'Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: Peptide isolation and cloning of a cDNA ' cited in the application see abstract see page 3953, right column, paragraph 5 - page 3955, right column, paragraph 1 see page 3955, right column, paragraph 3 - page 3956, left column, paragraph 4 ---	1-5,7-15
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 9, 25 March 1993 MD US, pages 6641-6648, MICHAEL E. SELSTED ET AL. 'Purification, primary structures, and antibacterial activities of Beta-defensins, a new family of antimicrobial peptides from bovine neutrophils' cited in the application see abstract see page 6643, right column, paragraph 2 - page 6646, left column, paragraph 1 see page 6647, left column, paragraph 2 - right column, paragraph 2 ---	1-5,7-15
P,X	SCIENCE (WASHINGTON, D. C.) , 267(5204), 1645-8 CODEN: SCIEAS;ISSN: 0036-8075, 17 March 1995 SCHONWETTER, BARRY S. ET AL 'Epithelial antibiotics induced at sites of inflammation' see page 1645, right column, paragraph 3 - page 1646, right column, paragraph 2 see page 1647, left column, paragraph 2 - right column, paragraph 2 -----	1-5,7-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte[illegible]al Application No
[illegible]/US 95/06761

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